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Carcinogenesis vol.13 no.7 pp.1269–1272, 1992

SHORT COMMUNICATION

Increased 8-oxodeoxyguanosine levels in lung DNA of A/J mice and F344 rats treated with the tobacco-specific nitrosamine 4-(methylnitrosamine)-1-(3-pyridyl)-1-butanone

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Evidence for the involvement of free radicals in nitrosamine carcinogenesis comes mainly from increased lipid peroxidation as a result of nitrosamine treatment. More direct evidence for nitrosamine-induced oxidative DNA damage has been lacking. In this study we examined the levels of 8-oxodeoxyguanosine or 8-hydroxydeoxyguanosine (8-OH-dG) in tissue DNA of mice and rats treated with the tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Multiple doses of NNK (0.25 or 0.50 mg/mouse, 3 times weekly for 3 weeks) administered by gavage resulted in a significant elevation of 8-OH-dG in lung DNA, from 2.1 to 3.8 adducts/10^5 dG for the lower dose or to 6.6 adducts/10^5 dG for the higher dose, 2 h after the last NNK administration. A single dose treatment of NNK by gavage (4 mg/mouse) also resulted in an increase of this lesion in the lung DNA, however, the increase was not statistically significant. In liver, however, the increase was only significant by multiple doses at the higher dose, from 2.3 to 3.4 adducts/10^5 dG. This lesion appeared to be repaired efficiently. At 4 and 24 h after NNK treatment, the 8-OH-dG levels declined to the basal levels in both liver and lung. A single dose of NNK (20 mg/rat) also caused a significant increase of 8-OH-dG from 3.0 to 5.1 adducts/10^5 dG in rat lung DNA. An increase of 8-OH-dG in liver DNA was also seen, however, it was not statistically significant. Unlike the liver and the lung, the 8-OH-dG levels in rat kidney, a non-target tissue, were inert to NNK treatment. These results provide for the first time direct evidence supporting the role of oxidative DNA damage in NNK lung tumorigenesis.

The tobacco-specific nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK*) induces lung cancer in various animal species (1,2). The specificity and potency of NNK in the induction of lung tumors suggest that it may be involved in lung cancer caused by smoking (2). Upon metabolic activation by cytochrome P450, NNK is converted into a methylating and a pyridyloxobutylating agent both of which alkylate DNA (3). Several studies showed that O^6-methylguanine (O^6-mG) is a critical lesion in NNK lung tumorigenesis (4–6). We have shown that the extent of inhibition of NNK-induced lung tumorigenesis in A/J mice by certain arylalkyl isothiocyanates was correlated with the inhibition of NNK-induced O^6-mG in lungs (7). These results further support the importance of DNA methylation by NNK in lung tumorigenesis.

*Abbreviations: NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone; O^6-mG, O^6-methylguanine; 8-OH-dG, 8-hydroxydeoxyguanosine.

Pyridyloxobutylation also occurs in animals treated with NNK, however, less is known about its role in NNK tumorigenesis (3,5). While the significance of DNA alkylation in nitrosamine carcinogenesis is widely accepted, ample evidence also indicates that DNA alkylation alone is not sufficient to account for its target-organ specificity (8). Recent studies in our laboratory showed that while lung tumor formation induced by multiple doses of NNK in A/J mice was inhibited by green tea, O^6-mG in lung DNA remained unchanged by these treatments (9). These results suggest that additional mechanism(s) other than DNA methylation may be involved in NNK lung tumorigenesis. Lipid peroxidation was induced in animals treated with nitrosamines (10,11). Studies also showed that antioxidants such as the polyphenol (−)-epigallocatechin gallate of green tea, ellagic acid and butylated hydroxyanisole were inhibitory against NNK-induced lung tumorigenesis in A/J mice (9,12). These results suggest that the formation of oxygen-derived free radicals is involved in NNK-induced lung tumorigenesis. However, direct evidence of a free-radical-mediated DNA lesion in nitrosamine-treated animals has been lacking. In this study we found an increase of 8-hydroxydeoxyguanosine (8-OH-dG), a common DNA adduct derived from reactive oxygen species, in lungs of mice and rats treated with NNK.

Two groups of 15 6-week old female A/J mice (Jackson Labs, Bar Harbor, ME) were treated with NNK in water (12.5 and

Table I. Levels of 8-OH-dG in lung DNA of mice treated with or without NNK[a]

NNK treatment (mg/mouse)	Hours after NNK treatment		
	2	4	24
None (control)	2.1 ± 1.1[b]	ND[c]	ND
0.25 (×9 doses)	3.8 ± 1.2[d]	1.8 ± 0.9	1.6 ± 0.22
0.50 (×9 doses)	6.6 ± 3.8[e]	1.7 ± 0.3	2.00 ± 0.7
4 (×1 dose)	4.1 ± 2.5	1.9 ± 0.3	3.0 ± 1.1

[a]Numbers are adducts/10^5 dG.
[b]Mean ± SD from five mice.
[c]Not determined.
[d]Significantly different from the control, $P < 0.05$.
[e]Significantly different from the control, $P < 0.01$.

Table II. Levels of 8-OH-dG in liver DNA of mice treated with or without NNK[a]

NNK treatment (mg/mouse)	Hours after NNK treatment		
	2	4	24
None (control)	2.3 ± 0.2[b]	ND[c]	ND
0.25 (×9 doses)	2.9 ± 0.6	2.3 ± 0.2	2.4 ± 0.6
0.50 (×9 doses)	3.4 ± 0.8[d]	2.5 ± 0.5	2.2 ± 0.5
4 (×1 dose)	3.0 ± 0.9	2.4 ± 0.5	2.1 ± 0.6

[a]Numbers are adducts/10^5 dG.
[b]Mean ± SD from five mice.
[c]Not determined.
[d]Significantly different from the control, $P < 0.05$.

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Table III. Levels of O^6-mG in liver and lung of mice 2, 4 and 24 h after NNK treatment

NNK treatment (mg/mouse)	O^6-mG (μmol/mol G)					
	Liver			Lung		
	2	4	24	2	4	24
0.25 (×9 doses)	32.1 ± 16.3[a]	78.4 ± 21.3	38.4 ± 17.1	11.9 ± 3.1	20.2 ± 5.8	11.0 ± 1.9
0.50 (×9 doses)	91.3 ± 22.2	101.4 ± 36.0	59.6 ± 7.2	24.1 ± 7.3	26.8 ± 8.1	22.0 ± 3.2
4 (×1 dose)	254.0 ± 25.9	354.5 ± 35.0	549.9 ± 91.5	27.0 ± 7.1	73.2 ± 5.6	73.4 ± 12.4

[a]Mean ± SD from five mice.

25.0 mg/kg body wt or −0.25 and 0.50 mg/dose/mouse) by gavage 3 times weekly for 3 weeks. A third group of 15 mice was given a single dose of NNK in water (4 mg/mouse or 200 mg/kg body wt) by gavage. Two, 4 and 24 h after the last NNK treatment, five mice from each group were killed and the lungs and livers removed for DNA isolation. For controls, five mice were given only water by gavage 3 times for 3 weeks and killed 2 h after the last gavage. In the rat study, 15 male F344 rats (Charles River, Kingston) were administered by i.p. a single dose of NNK in saline (25 mg/rat or 100 mg/kg body wt). Two, 4 or 24 h after NNK administration, five rats were killed at each time point. For controls, six rats were given a single injection of saline by i.p. Three rats were killed at 2 or 24 h after NNK injection. Liver, lung and kidney were removed for DNA isolation.

DNA was isolated by a modified Marmur's method (13). The precipitated DNA was kept at −20°C overnight and was solubilized in 0.5 ml (lung) or 0.6 ml (liver) of 0.01 M Tris−HCl buffer (pH 7.0). For 8-OH-dG analysis 10 μl of 0.5 M sodium acetate buffer (pH 5.1) was added to 0.2 ml of the DNA solution which was denatured at 90°C for 2 min. A 0.2 ml aliquot containing 100−200 μg DNA was treated with 10 U of nuclease P1 and incubated in the dark at 37°C for 30 min. After the addition of 40 μl of 0.4 M Tris−HCl buffer (pH 7.5) and 8 U of alkaline phosphatase, the mixture was incubated at 37°C for 1 h. The resulting hydrolysates were centrifuged and the supernatant was kept at 0°C for <5 h prior to the analysis of 8-OH-dG using a HPLC system coupled to an electrochemical detector following a published method (14). For O^6-mG analysis, a portion of the DNA solution was hydrolyzed in 0.1 N HCl at 80°C for 60 min and the resulting hydrolysate was analyzed by a HPLC-fluorescence detection method described previously (15). Statistical significance was determined by Student's *t*-test.

Our earlier study showed that mice given NNK by gavage 3 times weekly at a dose of 0.25 mg/mouse for 10 weeks developed an average of 22.5 lung tumors/mouse (9). In the present study, mice were given NNK by gavage 3 times weekly for 3 weeks at 0.25 mg, 0.50 mg/mouse or by a single dose of 4 mg/mouse. The single dose is comparable to the total dose received at 0.50 mg/mouse of NNK by this regimen. Table I shows that, 2 h after the last NNK treatment, levels of 8-OH-dG in mouse lung are significantly elevated in the treated groups as compared with those in the control group. We observed an increase of 8-OH-dG from 2.1 to $3.8/10^5$ dG at the low dose or $6.6/10^5$ dG at the high dose. Although the increases appeared to be dose-dependent, 8-OH-dG levels between the low and the high dose group are not statistically different. The single dose treatment also resulted in an increase of this lesion in the lung DNA, however, the increase is not statistically significant. The levels of 8-OH-dG in lungs quickly declined to the basal levels 4 h after NNK treatment and remained at those levels after 24 h. In the mouse liver, an increase of oxidative DNA damage was also observed upon NNK treatment (Table II). However, this increase was significant only when multiple high doses of NNK were given. As in the lung, the elevation of 8-OH-dG in the liver was only detected 2 h after the last NNK treatment. These results suggest that 8-OH-dG induced by NNK seems to be efficiently repaired to the basal levels in these tissues. O^6-mG levels in the mouse liver and lung DNA obtained from the same experiments were also determined after NNK treatment. Table III shows a clear dose-dependent increase of O^6-mG in these tissues. At 24 h after NNK treatment the levels of O^6-mG declined in groups treated with smaller multiple doses, whereas it continued to increase in the group treated with a large single dose of NNK. These results

Table IV. 8-OH-dG levels in lung, liver and kidney of rats treated with or without NNK[a]

	Hours after treatment	Control	Treated
Lung	2	2.96 ± 1.09[b]	5.14 ± 1.21[d]
	4	ND[c]	2.42 ± 0.5[illegible]
	24	3.32 ± 0.96	2.28 ± 0.96
Liver	2	2.87 ± 0.41	4.30 ± 0.70
	4	ND	4.87 ± 2.41
	24	2.65 ± 0.56	2.57 ± 0.52
Kidney	2	4.78 ± 2.09	5.88 ± 1.59
	4	ND	5.44 ± 0.25
	24	4.26 ± 1.09	4.34 ± 0.57

[a]A single dose of NNK was administered i.p. at a dose of 100 mg/kg body wt. Numbers are adducts/10^5 dG.
[b]Mean ± SD from three or five animals.
[c]Not determined.
[d]Significantly different from the control $P < 0.05$.

Table V. Levels of O^6-mG in liver, lung and kidney of rats 2, 4 and 24 h after NNK treatment[a]

Tissue	O^6-mG (μmol/mol G)		
	2	4	24
Liver	293.0 ± 101.2[b]	605.2 ± 94.2	450.8 ± 73.0
Lung	14.0 ± 8.1	50.5 ± 24.3	47.8 ± 31.2
Kidney	ND	16.4 ± 6.1	8.8 ± 6.0

[a]A single dose of NNK was administered i.p. at a dose of 100 mg/kg body wt.
[b]Mean ± SD from four rats.

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suggest that the repair enzyme of O^6-mG was somewhat impaired after a single large dose of NNK. Interestingly, while NNK-induced O^6-mG levels were at least 3-fold greater in the liver than in the lung at any given time point examined except for that at 2 h after the single dose, the increase of 8-OH-dG formation caused by NNK was more pronounced in the lung than in the liver. The protection of liver DNA against NNK-induced oxidative damage could be attributed to the greater activity in the liver than in the lung of antioxidant enzymes such as superoxide dimutase or glutathione peroxidase in rodents (16,17).

A single NNK dose given to rats i.p. also caused an increase of 8-OH-dG levels in both lung and liver DNA 2 h after treatment. As shown in Table IV, 2 h after NNK treatment the levels of this lesion in lung are significantly higher than the basal levels, however, the increase in the liver DNA is not statistically different from that found in the untreated group. Again, the elevated 8-OH-dG levels descended to basal levels 4 h after the NNK treatment. Unlike the lung and liver, rat kidney, a non-target tissue of NNK carcinogenesis was inert to the oxidative damage induced by NNK. The formation of O^6-mG in liver, lung and kidney of NNK-treated rats was also examined (Table V). Similar to the results in mice, NNK treatment resulted in 10- to 20-fold greater O^6-mG levels in the liver than in the lung at the time intervals examined; however, the extent of oxidative DNA damage caused by NNK in both tissues was comparable.

Thus far evidence for the potential role of free radicals in nitrosamine carcinogenesis comes mainly from the observations that treatment with nitrosamines such as *N*-nitrosodimethylamine, enhanced lipid peroxidation (18). To the best of our knowledge the present study is the first one to demonstrate that specific oxidative damage in the target tissue DNA was elevated upon nitrosamine treatment. Because 8-OH-dG is formed most probably from hydroxyl radicals, our results provide direct evidence supporting the association of free radicals in nitrosamine carcinogenesis. There are at least two plausible pathways from which free radicals could be generated during nitrosamine metabolism, e.g. cytochrome P450-dependent α-hydroxylation and reductive denitrosation (19–21). The present findings support a preliminary observation in humans which showed higher levels of 8-OH-dG in smoker's lung DNA than in non-smoker's (22). The increase in smokers could not be solely induced by NNK since many cigarette components other than NNK are also likely to cause oxidative damage (23,24). Furthermore, we found that 8-OH-dG was repaired efficiently in tissues, findings consistent with the results shown by others (25). The biological significance of 8-OH-dG in tumorigenesis is not clear. Nevertheless, recent studies have implicated elevated 8-OH-dG levels in hepatocarcinogenesis of 2-nitropropane, and renal carcinogenesis of diethylstilbesterol and potassium bromate (14,26,27). It is believed that free-radical-mediated oxidative damage is commonly associated with tumor promotion, although it is also possible that 8-OH-dG, a miscoding lesion, is involved in tumor initiation (28–32). The actual role of 8-OH-dG in NNK carcinogenesis awaits further studies. Regardless of the mechanism(s), our findings that 8-OH-dG formation increased in lungs of both mice and rats treated with NNK, suggest that, in addition to DNA alkylation, oxidative DNA damage may also contribute to the carcinogenesis of NNK.

Acknowledgements

We thank Dr Emerich Fiala for providing the 8-OH-dG standard and Shantu Amin for synthesizing NNK. This work was supported partially by grants CA-46535 and CA-51830 from the National Cancer Institute and a fellowship from the Institute of Nutrition and Food Hygiene, Chinese Academy of Preventive Medicine, Beijing, China.

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Received on January 21, 1992; revised on March 19, 1992; acepted on March 26, 1992

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